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Original Article

Cotton leaf curl Multan betasatellite impaired ToLCNDV ability to maintain cotton leaf curl Multan alphasatellite

O enrolamento da folha de algodão Multan betassatélite prejudica a capacidade de ToLCNDV de manter o enrolamento da folha de algodão alfassatélite Multan

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Abstract

Alphasatellites (family *Alphasatellitidae*) are circular, single-stranded (ss) DNA molecules of ~1350 nucleotide in size that have been characterized in both the Old and New Worlds. Alphasatellites have inherent ability to self-replicate, which is accomplished by a single protein, replication-associated protein (*Rep*). Although the precise function of alphasatellite is yet unknown, and these consider dispensable for infectivity, however, their *Rep* protein functions as a suppressor of host defence. While alphasatellites are most frequently associated with begomoviruses, particularly with monopartite than bipartite begomoviruses, they have recently been found associated with mastreviruses. The *in planta* maintenance of alphasatellites by helper geminivirus is still an enigma, with no available study on the topic. This study aimed to investigate whether a widely distributed bipartite begomovirus, tomato leaf curl New Delhi virus (ToLCNDV), can maintain cotton leaf curl Multan alphasatellite (CLCuMuA) in the presence or absence of cotton leaf curl Multan betasatellite (CLCuMuB). The findings of this study demonstrated that ToLCNDV or its DNA A could maintain CLCuMuA in *Nicotiana benthamiana* plants. However, the presence of CLCuMuB interferes with the maintenance of CLCuMuA, and mutations in the CP of ToLCNDV further reduces it. Our study highlighted that the maintenance of alphasatellites is impaired in the presence of a betasatellite by ToLCNDV. Further investigation is needed to unravel all the interactions between a helper virus and an alphasatellites.

Keywords: begomovirus, cotton leaf curl Multan alphasatellite, cotton leaf curl Multan betasatellite, maintenance, tomato leaf curl New Delhi virus.

Resumo

Alfassatélites (família *Alphasatellitidae*) são moléculas de DNA circulares de fita simples (ss) de ~1350 nucleotídeos de tamanho, que foram caracterizadas tanto no Velho como no Novo Mundo. Os alfassatélites têm capacidade inerente de autorreplicação, o que é realizado por uma única proteína, a proteína associada à replicação (*Rep*). Embora a função precisa dos alfassatélites ainda seja desconhecida, e estes sejam considerados dispensáveis para infectividade, entretanto, sua proteína Rep funciona como supressora da defesa do hospedeiro. Embora os alfassatélites sejam mais frequentemente associados a begomovírus, particularmente com begomovírus monopartidos do que bipartidos, eles foram encontrados recentemente associados a mastrevírus. A manutenção in planta de alfassatélites por helper geminivirus ainda é um enigma, sem estudos disponíveis sobre o tema. Este estudo teve como objetivo investigar se um begomovírus bipartido amplamente distribuído, o tomate leaf curl New Delhi virus (ToLCNDV), pode manter o alfassatélite Multan do enrolamento das folhas de algodão (CLCuMuA) na presença ou ausência do betassatélite Multan do enrolamento das folhas de algodão (CLCuMuB). Os achados deste estudo demonstraram que ToLCNDV ou seu DNA A poderia manter CL CuMuA em plantas de *Nicotiana benthamiana*. No entanto, a presença de CLCuMuB interfere na manutenção de CLCuMuA, e mutações no CP de ToLCNDV a reduzem ainda mais. Nosso estudo destacou que a manutenção de alfassatélites é prejudicada na presença de um betassatélite por ToLCNDV. Mais investigações são necessárias para desvendar todas as interações entre um vírus auxiliar e um alfassatélite.

Palavras-chave: begomovírus, enrolamento da folha de algodão Multan alfassatélite, enrolamento da folha de algodão Multan betasatélite, manutenção, tomato leaf curl New Delhi virus.

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1. Introduction

Alphasatellites belong to the family *Alphasatellitidae* (sub-family *Geminialphasatellitinae*), and have a circular, single-stranded (ss) DNA genome of ~1350 nucleotides length (Briddon et al., 2018; Varsani et al., 2021). Alphasatellites encode a single protein called *replication-associated protein* (*Rep*) in virion-sense, which mediates replication of alphasatellite, and thus alphasatellites are referred to as "satellite-like" molecules (Palukaitis et al., 2008). Additionally, alphasatellites have an adenine-rich region and a hairpin loop (nonanulceotide TAGTATT/AC) structure. Though alphasatellites are self-replicating molecules, they are encapsidated and moved by their helper virus. No particular role has been accredited to alphasatellites to date, and they are not required for symptomatic infection or disease development. On the other hand, alphasatellite attenuates symptoms in plants infected with begomovirus–betasatellite complexes by regulating the DNA titers of virus and/or betasatellite (Wu and Zhou, 2005). However, the *Rep* protein encoded by a few alphasatellites can suppress the host plant's gene silencing mechanism at the post-transcriptional level (Abbas et al., 2019; Zhao et al., 2022).

Alphastaellites are typically associated with begomoviruses, more frequently with monopartite than bipartite begomoviruses; however, they have recently been found associated with mastreviruses (Rosario et al., 2013; Hamza et al., 2018). Alphasatellites have been found associated with begomoviruses-betasatellite complexes, however, this is not always the case, and they may not be associated with all begomovirus–betasatellite complexes (Briddon et al., 2004). Alphasatellites were discovered prior to betasatellites in the Old World (OW). Likewise, alphasatellites have been identified in the New World (NW) associated with bipartite begomoviruses (Paprotka et al., 2010; Romay et al., 2010). Although the origin of alphasatellites is vague, nanoviruses are thought to be their ancestors as they exhibit a high degree of structural resemblance to nanoviruses. It is thought that alphasatellites were acquired by a begomovirus during a mix infection with a nanovirus (Mansoor et al., 2003; Mansoor et al., 1999; Wu and Zhou, 2005).

All begomoviruses (family *Geminiviridae*) are important phytopathogens with a circular and ssDNA genome encapsidated in a characteristic geminate-shaped particle. Members of this genus are transmitted exclusively by whitefly (*Bemisia tabaci*) in a circulative and persistent manner and are widely distributed in cultivated and non-cultivated plant species (Rojas et al., 2018; Barboza et al., 2019; Ijaz et al., 2024). All begomoviruses found in the NW are usually bipartite and both genome components are essential for symptomatic infection. Whereas in the OW, a prevalent majority of begomoviruses are monopartite and have a single genome component (a homolog of DNA-A of bipartite begomoviruses), however, only a few bipartite begomoviruses are known to exist in the OW.

ToLCNDV is a most prevalent and widespread bipartite begomovirus that poses a significant constraint to the production of many economically important crops, particularly tomato, in South and South-east Asia (Padidam et al., 1995) and has extended its geographic range to Africa and Europe (Mizutani et al., 2011; Juarez et al., 2014; Mnari-Hattab et al., 2015; Fortes et al., 2016). ToLCNDV genome encode six genes in both virion- and complementary-sense. The coat protein (CP) and (A)V2 protein are encoded by the virion-strand, whereas the replication-associated protein (Rep), the (A)C2 protein (also known as the transcriptional activator protein [TrAP]), the replication-enhancer protein (REn), and the (A)C4 protein are encoded by the complementary-sense strand. The functions and activities of these proteins have been thoroughly studied (Fondong, 2013; Hanley-Bowdoin et al., 2013). The DNA B of ToLCNDV encodes two movement protein, one in each orientation, nuclear shuttle protein (NSP) in the virion-sense and the movement protein (MP) in the complementary-sense (Sanderfoot and Lazarowitz, 1995). ToLCNDV has been isolated from a wide variety of plants, including bitter gourd, bottle gourd, chilli, cucumber, cotton, muskmelon, tomato, okra and a variety of weeds (Hussain et al., 2004; Tahir and Haider, 2005; Haider et al., 2006; Ito et al., 2008; Akhter et al., 2009; Mizutani et al., 2011). Both components of ToLCNDV are required for symptomatic infection. However, its DNA-A alone can infect plants, albeit at a reduced rate and without causing symptoms after *Agrobacterium* inoculation (Padidam et al., 1995; Iqbal et al., 2017). ToLCNDV is found to be associated with CLCuMuB in natural infection in Pakistan and India (Jyothsna et al., 2013; Hameed et al., 2017).

We previously demonstrated the trans-replication and maintenance of betasatellite by two different begomoviruses (Iqbal et al., 2012; Iqbal et al., 2017), implying that the interaction and association of betasatellites with their helper begomoviruses is more complex than just trans-replication and movement. The current study is a continuation of the same series that investigates the maintenance of alphasatellite in the presence and absence of a betasatellite. To investigate this notion, a bipartite begomovirus, ToLCNDV, and the cotton leaf curl Multan alphasatellite (Cα) were co-inoculated into *Nicotiana benthamiana* (Nb) plants. Additionally, the cotton leaf curl Multan betasatellite was also included to decipher its role in the begomovirus–alphasatellite complex.

2. Materials and Methods

2.1. Production of ToLCNDV and DNA-satellites constructs for Agrobacterium-mediated inoculation

The study used the ToLCNDV DNA A (Acc. # U15015) and DNA B (Acc. # U15017) clones (Padidam et al., 1995). The CP gene mutant of ToLCNDV was constructed using a designed set of mutational and back-to-back primers (Table 1), the initial full-length amplicon of CP mutant was cloned into pTZ57R and sequenced completely to ensure any unwanted mutation. The construction of a complete infectious dimeric construct of ToLCNDV ($TA^{\Delta CP}$) bearing a mutation of the CP gene has been described earlier (Iqbal et al., 2017).

The construction of infectious clone of cotton leaf curl Multan alphasatellite (Cα; Acc. # AJ132344) (Mansoor et al.,

Table 1. Oligonucleotide primers, their sequence, and targets were used in the study.

Primer Name	Sequence (5'-3')	Comments*
ToLNDmCPF	ATCGATTAGGGTAGCGATTCTaGTGTG	To mutate CP
ToLNDmCPR	ATCGATCGCGATGTGTGAGTCCAGTTC	
ToLNC2pvx/35 F	CAAGTCGACATGCAGTCTTCATC	Detection of DNA-A of ToLCNDV
ToLNC2pvx/35 R	ATCCCGGGACTTAAGGACCTGG	
DNA101	CTGCAGATAATGTAGCTTACCAG	Detection of alphasatellite
DNA102	CTGCAGATCCTCCACGTGTATAG	
Rep09(MuAOld) F	TAAAAGCTTATGCCTACTATTCAGTCACAATG	Amplification of Alpha-Rep
Rep09(MuAOld) R	TTAGGATCCTTATTCCATATATTCGCCACAC	
BetaC1F	ATAAATCGATATGACAACGAGCGGAACAAA	Detection of betasatellite
BetaC1R	TGTTCCCGGGTTAAACGGTGAACTTTTATT	
Begomo qPCR F 1	ATGTGGGATCCACTGTTAAATGAGTTCCC	Quantification of TA through qPCR
Begomo qPCR R 1	GATTATATCTGCTGGTCGCTTCGACATAA	
Beta qPCR F 2	CAAGTATATCAAGTCTGTGAACTATATCTT	Quantification of betasatellite through qPCR
Beta qPCR R 2	GATACTATCCACAAAGTCACCATCGCTAAT	
ALPHA qPCR F 2	ATTCAAATTTCAAATTTGAAATCTTGGCA	Quantification of alphasatellite through qPCR
ALPHA qPCR R 2	CCTTCTTATCACGAGGGATATCAAATACAA	
Tr03 F	TCTGCCCTATCAACTTTCGATGGTA	18s eukaryotic rDNA
Tr04R	AATTTGCGCGCCTGCTGCCTTCCTT	

*Lowercase nucleotides indicated that an additional nucleotide was added to cause a frameshift mutation, while underlined nucleotides indicated that a nucleotide substitution inserted a stop codon.

1999; Shahid, 2009) and CLCuMuB (Cβ: (Saeed et al., 2005) have been described previously.

2.2. Agrobacterium-mediated inoculation of N. benthamiana plants

All the binary vector constructs used in the study were electroporated into Agrobacterium tumefaciens strain LBA4404 and GV3101. *A. tumefaciens* inocula harboring binary vector constructs of ToLCNDV and DNA-satellites were prepared and mixed in equal proportions to an OD of 0.5 ($Abs_{650\ nm}$). Nb plants, at the 5-6 leaf stage, were inoculated (0.5-1 mL) with a sterile syringe on the adaxial side of three leaves, and the inoculated plants were maintained in an insect free glasshouse as described earlier (Iqbal et al., 2021). At 25 days of post-inoculation (dpi), symptoms were noted and recorded with a digital camera, and leaf samples were collected for subsequent analysis.

2.3. PCR-mediated detection of TA, Cα and Cβ

Total genomic DNA was extracted from the newly emerging leaves of the inoculated plants at 25 dpi (Doyle and Doyle, 1990). The extracted DNAs were purified, their quality and quantities were determined spectrophotometrically (SmartSpec 300 UV/Vis, Bio-Rad), and then used in the subsequent analyses. ToLNC2pvx/35 F/R primers were used to detect ToLCNDV DNA A (TA), DNA101/DNA102 primers for Cα and β01/ β02 primers for Cβ detection (Table 1). Approximately 10 µg of the isolated nucleic acid was electrophoresed in a pre-stained 1.5% agarose gel as described previously (Iqbal et al., 2017). Detection of TA, Cα and Cβ was achieved in Southern blotting using PCR-amplified digoxigenin (DIG)-labeled probes, as previously described (Iqbal et al., 2017). The DIG-labelled probes for TA, Cα and Cβ detection were synthesized via PCR using the primer pairs ToLNC2pvx-35sF/ToLNC2pvx-35sR, Rep09(MuAOld)F/Rep09(MuAOld)R, and BetaC1F/BetaC1R, respectively (Table 1). The transfer of DNA and hybridization were carried out as described earlier (Sambrook et al., 1989), the signals were detected on X-ray film (Super RX, Fuji film) treating with CDP-Star (Roche, Germany).

2.4. Absolute quantification of TA, Cβ, and Cα

To quantify the viral load and associated DNA satellites in selected Nb plants, especially those from which diagnostic PCR revealed the presence of viral components, the quality, and quantity of isolated genomic DNAs were assessed spectrophotometrically (NanoDrop ND-1000). Absolute quantification of TA, Cα and Cβ was accomplished by serially diluting the standards (viral monomeric clones) tenfold at concentrations ranging from 20 ng-to-0.002 ng (Shafiq et al., 2017). Absolute quantification was performed using a thermocycler on three independent replicates of each sample (Bio Rad iQTM5 thermocycler). Three technical repeats were used to calculate the standard error for each value. In each run, a reaction (96-well) plate was loaded with the positive control (an individual respective viral clone), a negative control (ultra-pure water)

and a housekeeping gene (18s rDNA) to normalize DNA concentration differences between samples (Allmann et al., 1993). The titer of viral components was determined as "ng per µg of the genomic DNA" and will be mentioned only in "ng" in the subsequent sections. The PCR conditions and reagents concentrations have already been described (Shafiq et al., 2017).

2.5. Data and statistical analysis

To quantify each virus component, an independent standard curve of each component was generated by plotting the Ct value against the total amount of DNA using linear regression analysis (Shafiq et al., 2017). Analysis of variance (ANOVA) was used to determine the differences in infectivity. The least significant difference (LSD) at P ≤ 0.05 in the R program was calculated using a pairwise comparison (R Development Core Team, 2016).

3. Results

3.1. Inoculation of Nb plants with ToLCNDV and DNA satellites

Agrobacterium-mediated inoculation of ToLCNDV (TA and TB) induced severe infection in Nb plants and all the inoculated plants exhibited vein thickening and leaf curling (upward) symptoms at 12 dpi (Figure 1; Table 2). The induced symptoms progressed subsequently to severe stunting in growth as compared to non-inoculated plants (Figure 1). This infection was readily detectable in the

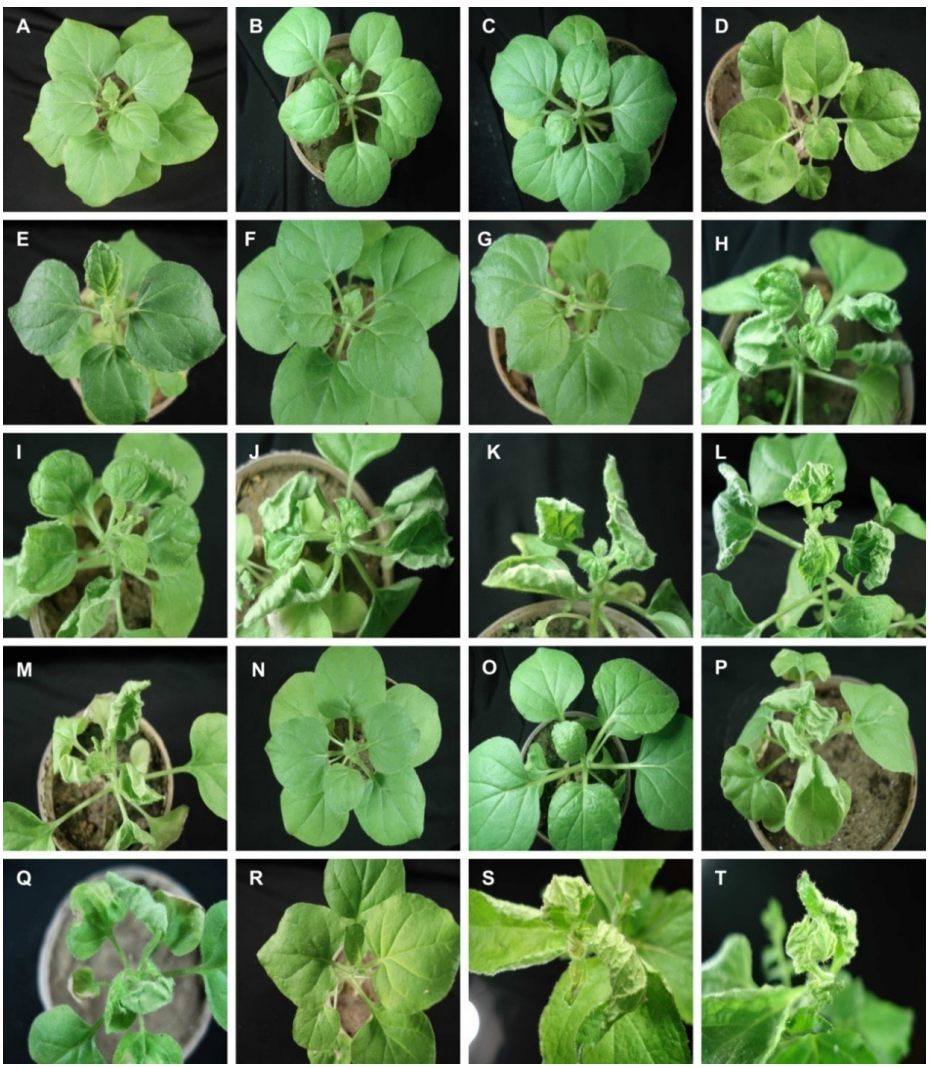

Figure 1. Symptoms exhibited by Nb plants upon inoculation with ToLCNDV, its CP gene mutant, along with DNA satellites. The shown Nb plants were either un-inoculated (healthy; A) or inoculated with TA (B), $TA^{\Delta CP}$ (C), TA and Cα (D), $TA^{\Delta CP}$ and Cα (E), TA and Cβ (F), $TA^{\Delta CP}$ and Cβ (G), TA and TB (H), $TA^{\Delta CP}$ and TB (I), TA, TB and Cβ (J), $TA^{\Delta CP}$, TB and Cβ (K), TA, TB and Cα (L), $TA^{\Delta CP}$, TB and Cα (M), TA, Cβ and Cα (N), $TA^{\Delta CP}$, Cβ and Cα (O), TA, TB, Cβ and Cα (P), $TA^{\Delta CP}$, TB, Cβ and Cα (Q), TA and Cβ (before inoculation of TB; R), TA and Cβ (after inoculation with TB; S), TA and Cα (before inoculation (D) and after inoculation with TB (T). Photographs from panel A-to-R were taken at 25 dpi, whereas photographs S and T were taken at 40 dpi.

Table 2. ToLCNDV infectivity and symptoms in *N. benthamiana* plants when inoculated alone or with DNA satellites.

Inoculum*	Infectivity									Southern blot Analysis****			Symptoms *****	Latent period (days)
	PCR diagnostics** (plants infected/ inoculated)			Infectivity (%)			Significance***							
	TA	Cα	Cβ	TA	Cα	Cβ	TA	Cα	Cβ	TA	Cα	Cβ		
Mock	0/9	0/9	0/9	0/9	0/9	0/9	A	--	--					
TA	6/15	--	--	40	--	--	CD	F	J	ND	--	--	NS	--
$TA^{\Delta CP}$	1/15	--	--	6.67	--	--	E	H	J	ND	--	--		--
TA, Cα	5/15	3/15	--	33.33	20	--	DE	FG	J	ND	ND	--	NS	--
$TA^{\Delta CP}$, Cα	1/15	0/15	--	6.67	0	--	E	H	J	ND	ND	--	NS	--
TA, Cβ	10/15	--	8/15	66.67	--	53.33	BC	H	I	ND	--	ND	NS	--
$TA^{\Delta CP}$, Cβ	6/15	--	5/15	40	--	33.33	CD	H	IG	ND	--	ND	NS	--
TA, TB	9/9	--	--	100	--	--	AB	H	J	D	--	--	LC,VT, ST	12
$TA^{\Delta CP}$, TB	9/9	--	--	100	--	--	AB	H	J	D	--	--	LC,VT, ST	12
TA, TB, Cβ	12/12	--	4/12	100	--	33.33	AB	H	G	D	--	ND	LC,VT, ST	10
$TA^{\Delta CP}$, TB, Cβ	12/12	--	3/12	100	--	25	AB	H	G	D	--	ND	LC,VT, ST	12
TA, TB, Cα	12/12	3/12	--	100	25	--	AB	F	J	D	--	--	LC,VT, ST	12
$TA^{\Delta CP}$, TB, Cα	12/12	2/12	--	100	16.67	--	AB	FGH	J	D	ND	--	LC,VT, ST	13
TA, Cβ, Cα	10/15	1/15	9/15	66.67	6.67	60	CD	GH	I	D	ND	--	NS	--
$TA^{\Delta CP}$, Cβ, Cα	8/15	0/15	6/15	53.33	0	40	CD	H	IG	D	ND	ND	NS	--
TA, TB, Cβ, Cα	15/15	1/15	6/15	100	6.67	40	A	H	IG	D	ND	ND	LC, VT, ST	11
$TA^{\Delta CP}$, TB, Cβ, Cα	15/15	0/15	6/15	100	0	40	A	H	IG	D	ND	ND	LC, VT, ST	13-14

*The inoculated viral components are abbreviated as tomato leaf curl New Delhi virus DNA A (TA), DNA B (TB), TA having a CP gene mutation ($TA^{\Delta CP}$), cotton leaf curl Multan alphasatellite (Cα), and cotton leaf curl Multan betasatellite (Cβ); **Cumulative results of three separate experiments; ***Values denoted by distinct letters are statistically significantly different at $p \leq 0.05$. Values should be compared column-wise; ****Southern blot analysis detections are abbreviated as detected (D), not detected (ND), not tested (--); *****Symptoms are abbreviated as leaf curling (LC), stunting (ST), vein thickening (VT), or no symptoms (NS).

inoculated plants by PCR and Southern blotting (Table 2; Figure S1). The absolute quantification of TA using real-time qPCR revealed that its titer was 59 ng μg^{-1} of the genomic DNA (hereafter it will referred to as ng only) (Figure 2).

Co-inoculation of Nb plants with ToLCNDV and Cβ led to a symptomatic infection comparable to the infection induced by ToLCNDV alone (Table 2; Figure 1). Nonetheless, two distinct groups of plants with different latent periods were observed; the first group showed early onset of symptoms at 10 dpi, while the second group showed delayed onset of symptoms at 12 dpi. Subsequent PCR-based diagnostics showed that plants with a shorter (10 days) latent period contained Cβ, whereas those with a longer (12 days) latent period did not contain Cβ. In the diagnostic PCR, 4 of the 12 inoculated plants contained Cβ (Table 2). Southern blot hybridization did not detect the Cβ (data not shown), indicating that the Cβ DNA titer was below the detection threshold. No substantial difference in TA titers was observed between plants co-inoculated with Cβ and plants infected with the virus alone (Figure S1). In qPCR, however, the detected titer of TA was significantly higher (100.8 ng) and was almost doubled than obtained without Cβ (59 ng) (Figure 2).

TA alone could infect and asymptomatically spread in 6 (out of 15 inoculated) Nb plants (Figure 1; Table 2). Although no TA was detected using Southern blot hybridization, but qPCR data showed a significantly higher (57 ng) TA titer (Figure 2). Co-inoculation of TA and Cβ into Nb plants did not result in symptomatic infection (Figure 1). Southern blot hybridization of extracted DNA from TA/Cβ inoculated plants revealed no detection of either TA or Cβ, indicating that their titers were below the detection threshold (Figure S1). However, PCR-based diagnostics revealed TA in 10 plants (out of 15 inoculated), while Cβ was detected in 8 plants (Table 2). The plant shown to harbour TA and Cβ were used to quantify the TA and Cβ. The TA titer was determined to be 27.8 ng and the Cβ titer was 24.2 ng (Figure 2).

Co-inoculation of Nb plants with Cα and TA resulted in asymptomatic infection (Figure 1), with TA being detected in 5 of the 14 inoculated Nb plants, but Cα was detected only 3 plants (Table 2). The plant shown to contain TA and Cα by PCR were subjected to quantification and found to contain 55.2 ng of Cα (Figure 2). Although alphasatellite downregulated the level of helper virus, but notably an increased level of TA (95.2 ng) was found in qPCR as compared to TA alone (57 ng) (Figure 2).

The Cα was only maintained in plants when co-inoculated with TA or ToLCNDV (TA and TB). However, Cα presence did not affect infection symptoms or the

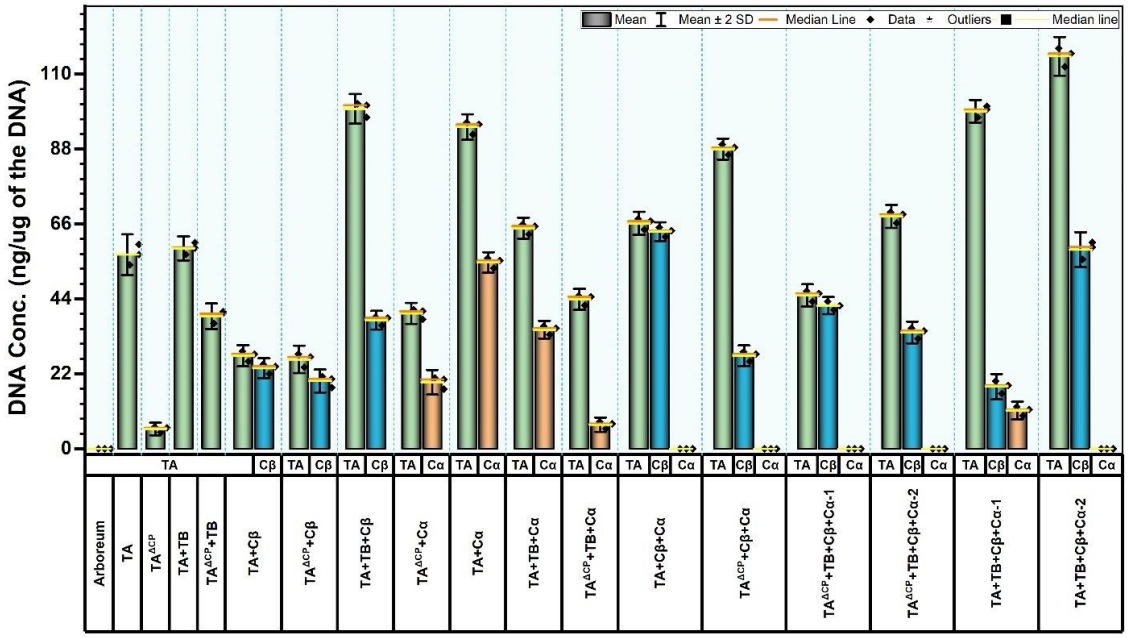

Figure 2. The absolute quantification of ToLCNDV and DNA satellites (alpha- and betasatellite) in Nb inoculated plants. The error bars indicate the standard deviation of three separate measurements. Abbreviations used are tomato leaf curl New Delhi virus DNA A (TA), DNA B (TB), TA having a CP gene mutation ($TA^{\Delta CP}$), cotton leaf curl Multan alphasatellite (Cα), and cotton leaf curl Multan betasatellite (Cβ).

latent period (Figure 1). Despite the virus higher titer (as determined by qPCR and Southern blotting for TA), the alphasatellite was only detected by PCR but not by Southern blotting (data not shown), indicating that Cα levels were low.

3.2. Co-inoculation of CP mutant of ToLCNDV with satellites

Nb plants inoculated with $TA^{\Delta CP}$ did not develop symptoms (Figure 1), however viral DNA was detected in only one plant (out of 15 inoculated) by PCR (Table 2), significantly less than in plants inoculated with TA (6 infected out of 15 inoculated) (Table 2). While no TA DNA was detected through Southern blot hybridization (Figure S1), but qPCR revealed that a plant contained TA DNA at a very minute concentration of 6.2 ng (Figure 2).

Co-inoculation of $TA^{\Delta CP}$ with Cβ induced no symptoms in Nb plants; however, PCR-based diagnostics detected both TA (in 6 of 15 inoculated plants) and Cβ (in 5 of 15 inoculated plants) in systemic leaves (Table 2). Quantification of isolated genomic DNA using qPCR confirmed the low titers of TA (27 ng) and Cβ (20.5 ng) (Figure 2). This infection was not detected in Southern blot hybridization (Figure S1).

Inoculation of Nb plants with $TA^{\Delta CP}$ and TB induced symptoms identical to those exhibited by the wild-type ToLCNDV, albeit with a slightly longer latent period, 13-14 days than 12 for ToLCNDV (Table 2). TA was detected in the newly emerging leaves of the symptomatic plants by PCR, qPCR and Southern blot hybridization (Figure 2; Figure S1). These plants were found to harbour a reduced quantity of TA DNA (39.7 ng) as compared to CP-intacted inoculated plants (59 ng) (Figure 2).

Co-inoculation of Nb plants with $TA^{\Delta CP}$, TB, and Cβ resulted in symptoms comparable to those induced by ToLCNDV (Figure 1). The only difference was the longer latent period (13 dpi) in comparison to plants inoculated with TA and TB, and Cβ (12 dpi). Although the levels of viral DNA ($TA^{\Delta CP}$ and TA) were almost comparable in Southern blot hybridization (Figure S1), but qPCR results revealed a slightly higher TA level (116 ng) than TA (100.8 ng) level in TA, TB, and Cβ inoculated plants (Figure 2). PCR-based diagnostics revealed that the CP mutation perturbed the ability of ToLCNDV to efficiently maintain the betasatellite, and Cβ was detected in three (out of 11 inoculated) plants (Table 2). In Southern blotting, only the virus was detected (Figure S1), not the betasatellite (data not shown).

Inoculation of $TA^{\Delta CP}$ with Cα into Nb plants did not develop symptoms, and the infection remained undetectable through Southern blot hybridization (Figure 1; Figure S1). Diagnostic PCR revealed that one plant (out of 14 inoculated) contained TA DNA but did not contain Cα (Table 2). Contrary to Southern blotting and PCR, qPCR detected both $TA^{\Delta CP}$ (40.4 ng) and Cα (20.4 ng) with reduced DNA levels (Figure 2).

Nb plants co-inoculated with $TA^{\Delta CP}$, TB, and Cα exhibited symptoms that were comparable to those exhibited by $TA^{\Delta CP}$/TB inoculated plants (Table 2). This infection was easily detectable through PCR, qPCR and Southern blot hybridization. The diagnostic PCR revealed TA in all inoculated plants; however, Cα was detected in only two (out of 12) plants (Table 2). The DNA level of $TA^{\Delta CP}$ was determined to be 44.6 ng using qPCR, while that of Cα was 7.4 ng (Figure 2).

3.3. Co-inoculation of both alpha- and betasatellite with ToLCNDV

Co-inoculation of Nb plants with all four viral components (TA, TB, Cα, and Cβ) or with three components (TA, Cα and Cβ) led to the maintenance of betasatellite in more plants but not the alphasatellite (Table 2). Although the TA, Cβ and Cα inoculated plant did not exhibit symptoms, but diagnostic PCR revealed the presence of TA in 10 plants, Cβ in 8 plants, and Cα in just one plant, out of 15 inoculated plants (Table 2). In Southern blot analysis, only TA could be detected (Figure S1), while both satellites remained undetectable (data not shown). TA and Cβ levels were relatively high in qPCR, measuring 66.8 and 64 ng, respectively. Nonetheless, Cα was not be detected and quantified (Figure 2).

The symptoms produced by Nb plants inoculated with TA, TB, Cβ, and Cα were indistinguishable from those produced by TA, TB inoculated plants; however, the principal difference was the earlier onset of symptoms at 11 dpi on some plants (Table 2). Again, two groups of plants with two different latent periods were observed. The PCR results showed that TA was present in 15 inoculated plants, Cβ was present in 6 plants, and Cα was present in only one plant (Table 2). Plants with shorter latest period contained the Cβ DNA, whereas plants with longer latent period did not. In qPCR, two different tested plants; one with higher Cβ (59.2 ng) titer contained higher TA titer (116 ng), while the other with lower Cβ (18.7 ng) titer contained a slightly lower amount (99.6 ng) of TA (Figure 2). The plant that tested positive for Cα by diagnostic PCR contained a very low titer of Cα (11.6 ng). Notably, the plant harboring Cα had a lower Cβ titer (18.6 ng) than a plant that did not harbor Cα, which had a higher Cβ titer (Figure 2).

3.4. Co-inoculation of both alpha- and betasatellite with CP mutant

None of the Nb plants that were inoculated with $TA^{\Delta CP}$ along with Cβ and Cα developed symptoms (Figure 1). However, diagnostic PCR revealed the presence of TA in 8 plants, Cβ in 6 plants, and Cα in just 2 plants out of 15 inoculated plants (Table 2). Only TA was detected in Southern blot hybridization but not Cβ or Cα, suggestive of low levels than the detection threshold (Figure S1). The qPCR results revealed a significantly higher titer of TA (177 ng) and Cβ (56 ng), whereas Cα could not be quantified in the plant that tested positive in diagnostic PCR (Figure 2).

All Nb plants inoculated with the $TA^{\Delta CP}$, TB, Cβ, and Cα developed infection symptoms comparable to those induced by wild-type ToLCNDV (Figure 1). However, a longer latent period, 13-14 days, was observed (Table 2). PCR-based diagnostic revealed the presence of virus in all (15 inoculated) plants, Cβ in 6 plants, and Cα could not be detected even from a single plant (Table 2). Southern blot hybridization showed that only TA was in the detection range, while DNA levels of both satellites were below the detection limit (Figure S1). The plant found to contain 45.6 ng and 34.62 ng of TA DNA, and 42 ng and 68.8 ng of Cβ, in 2 different tested plants, respectively (Figure 2). Again, Cα could not be quantified in these plants.

Interestingly, maintenance of alphasatellite was compromised in the presence of betasatellite, confirming the existence of some form of interference against satellite maintenance.

3.5. Inoculation of ToLCNDV DNA B into the newly emerging tissue of TA infected plants

Nb plants inoculated with TA alone or with Cα or Cβ did not develop symptoms and some of these plants were found to harbour TA DNA in diagnostics PCR and qPCR. To verify the presence of TA and which plants harboured it, newly emerging leaves of these plants were inoculated with TB. After 8-9 days of TB inoculation, only those Nb plants which harboured TA exhibited typical leaf curl and vein thickening symptoms, whereas the others remained asymptomatic (Figure 1S-1T). These infections were further confirmed by diagnostic PCR but not by Southern blotting and qPCR (data not shown).

4. Discussion

Although alphasatellites were discovered over a couple of decades ago (Saunders and Stanley, 1999), and their discovery resulted in the identification of betasatellites, but our understanding about these evolutionarily intriguing molecules is limited. Researchers have only recently begun a thorough investigation to unravel the diversity, structure, and functions of alphasatellites. Despite their ease of maintenance in experiments by curtoviruses and bipartite begomoviruses, as well as their transmission by leafhoppers in the presence of a curtovirus (Saunders et al., 2002), alphasatellites were initially found only in the OW along with monopartite begomoviruses (Briddon et al., 2004). Alphasatellites are quite diverse DNA satellites (Nawaz-ul-Rehman et al., 2012) and have been found in the NW along with bipartite begomoviruses (Paprotka et al., 2010; Romay et al., 2010; Jeske et al., 2014). Rep encoded by alphasatellites (at least for a few alphasatellites) functions as a suppressor of gene silencing activity (Nawaz-ul-Rehman et al., 2010; Rodriguez-Negrete et al., 2014; Abbas et al., 2019) – thus has a potential role in circumventing host defenses.

Alphasatellites continue to be an enigma because it is unclear what selective advantage they confer to their helper viruses during infection and how they are maintained during *planta* movement. Earlier research on the subject revealed that they could down-regulate virus replication, thereby attenuating symptoms, by competing for cellular resources (Saunders and Stanley, 1999), ensuring the survival of infected plants and enhancing the possibility of onward viral transmission. Some research in favour of this notion has been forthcoming (Idris et al., 2011), although the result of some studies, most notably of (Patil and Fauquet, 2010), were inconsistent. The findings discussed here refute this notion.

Perhaps the most surprising finding in this study was the low DNA levels of alphasatellite and their poor *in planta* maintenance, which further worsened in the presence of betasatellite. During the first cotton leaf curl disease (CLCuD) outbreak in Pakistan in the 1990s, all

plants infected with the begomovirus and betasatellite contained an alphasatellite (Amrao et al., 2010). The alphasatellite used in this study was from that era, as being the first isolated alphasatellite (Mansoor et al., 1999). Thus, one might anticipate the alphasatellite would maintain efficiently. Although these findings (poor maintenance of alphasatellite) are consistent with an earlier study that showed poor maintenance of an alphasatellite by a NW tomato golden mosaic virus (Saunders et al., 2002), but the contrast with another study that demonstrated relatively efficient maintenance of alphasatellite by an African cassava mosaic virus following mechanical inoculation (Saunders and Stanley, 1999). Another study has also revealed that a curtovirus (beat curly top virus) can efficiently maintain an alphasatellite but not a mastrevirus (BeYDV; (Saunders et al., 2002). There is thus no discernible or persistent trend regarding the factors affecting the maintenance of alphasatellites.

ToLCNDV, a virus that has been extremely informative in studies using a betasatellite (Iqbal et al., 2017), was an inappropriate choice due to its low DNA levels in the absence of the DNA B and upon CP gene mutation. Alternatively, the alphasatellite may lower betasatellite levels in the model system used here, interfering with component maintenance. Previous research demonstrating alphasatellite-mediated symptom amelioration has shown that alphasatellite substantially decreases betasatellite DNA level (Wu and Zhou, 2005; Idris et al., 2011). Since these studies used monopartite begomoviruses, while this study used a bipartite begomovirus, likely, the alphasatellite's "interference" with betasatellite replication or spread explains the apparent lack of alphasatellite maintenance. Further studies are needed to address this possibility. Certain alphasatellites have been shown to reduce symptoms and a slight reduction in betasatellite titer but not helper begomovirus titer (Kumar et al., 2017), whereas another study showed that alphasatellites not only attenuated symptoms but also reduced helper virus and betasatellite accumulation (Idris et al., 2011).

The two primary molecular methods for virus detection were the Southern blot and polymerase chain reaction (PCR). Both methods, however, have inherent limitations. Southern blotting, for example, is a lengthy and laborious procedure that requires a large amount of high-quality DNA. While PCR has a limitation in terms of end-point results, it is also time-consuming and produces size discrimination results; additionally, gels may not achieve higher resolution, mainly when the amplicon is small in amount. In comparison, qPCR has emerged as a viable alternative in molecular diagnostics because of several advantages, including its speed, low input DNA requirement, and wide dynamic range of accurate quantification. The difference in results obtained using qPCR versus Southern blot and conventional diagnostic PCR may be accredited to the high sensitivity of qPCR.

As a side note, TA infected asymptomatic plants were inoculated with DNA B to confirm the presence of ToLCNDV DNA-A. Such inoculations result in the complete ToLCNDV virus infection symptoms in the plants. The onset of symptomatic infection established the presence of TA in the newly emerging young leaf tissues of such asymptomatic plants and showed that DNA A component is capable of yielding typical infection when provided with homologous DNA B. These findings demonstrate that, in the absence of DNA B, ToLCNDV DNA-A is capable of successfully invading newly emerged tissues despite the presence of betasatellite.

The fact that alphasatellites are dispensable for geminivirus infection suggests that the advantage of having an alphasatellite is subtle. Nonetheless, the frequency in which alphasatellites are found along with begomoviruses and mastreviruses, in particular, suggests that the existence of alphasatellites confers a significant selective advantage, which may likely be conferred by circumventing the host defense via the TGS pathway. As self-replicating molecules, alphasatellites require host cellular factors for replication, putting them in direct competition with the helper virus and betasatellite.

5. Conclusions

Betasatellite interference with alphasatellite maintenance of by reducing its titer could be described in two ways: either the betasatellite interferes with the alphasatellite in order to increase its chances of replication, assembly and spread, or it conditions and directs the host cellular machinery toward viral replication, assembly and spread, thereby increasing its own survival. The cellular and molecular mechanisms underlying this hypothesis will be pretty intriguing and could be a future research topic.

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Supplementary Material

Supplementary material accompanies this paper.

Figure S1. ToLCNDV detection using Southern blot analysis in Nb plants. DNA extracts (~10 µg) resolved on the gel were of a mock-inoculated plant (M) and plants inoculated with TA (1), TA and Cα (2-3), TA and Cβ (4), $TA^{\Delta CP}$ and Cβ (5), $TA^{\Delta CP}$ and Cα (6), TA, Cβ and Cα (7-9), $TA^{\Delta CP}$, Cβ and Cα (10-12), TA, TB and Cα (13) $TA^{\Delta CP}$, TB and Cα (14), TA, TB, Cβ and Cα (15-16) and $TA^{\Delta CP}$, TB, Cβ and Cα (17-19). The sample in lane H was taken from an un-inoculated, healthy plant.

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